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Phosphoamidase Assay

10   ## Field of the invention

The present invention relates to the use of compounds and to methods for the detection, characterization and qualitative and/or quantitative determination of the activity of phosphoamidases or protein phosphoamidases specific for hydrolyzing phosphoamide (P-N) bonds of N-phosphorylated basic amino acids like

15   pholysine, phosphoarginine and phosphohistidine or of peptides comprising these phosphorylated amino acids.

Background of the invention

Communication between individual cells in multicellular organisms is essential for

20   the regulation and coordination of complex cellular processes such as growth, differentiation, migration and apoptosis. Signal transduction networks mediating these biological mechanisms use reversible protein phosphorylation as a fundamental tool. According to a modest estimation about one third of the known proteins can be phosphorylated in eukaryots. In the past decades it turned out

25   that a multitude of regulatory cycles are intimately interlocked through overlapping substrates and the mutual regulation of kinases and phosphatases via phosphorylation/dephosphorylation by other kinases and phosphatases, respectively (Cohen,P. (2000) *Trends Biochem. Sci.* **25**, 596-601). Deregulation of enzymatic dephosphorylation will cause substantial malfunctions and therefore

30   are causal for diseases.

Protein phosphatases are grouped according to their activity on the various phospho-amino acids, namely the most prominent phosphorylated amino acids serine (Ser), threonine (Thr) and tyrosine (Tyr). However, further amino acids

35   need to be considered as relevant phospho-derivatives, namely histidine (His), lysine (Lys) cysteine (Cys), aspartic acid (Asp), and glutamic acid (Glu). Systematic classification of kinases concern the nature of the phosphate accepting amino acids, e.g. whether a phosphate ester, thio ester, phospho-

5   amidate or phosphate carboxylate anhydride is formed (Matthews,H.R. & Chan,K. (2001) *Methods Mol. Biol.* **124:** 171-182).

The phospho-amino acids His, Asp, Glu, Lys and Cys show distinct chemical properties like acid-lability and faster physiological turnover which are commonly used for characterization (Sickmann,A. & Meyer,H.E. (2001) *Proteomics* **1**, 200-

10   206). Up to now the less abundant phospho-amino acids predominantly showed up in cellular processes of bacteria, plants (Chang,C. & Meyerowitz,E.M. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 4129-4133) and lower eucaryots (Huang,J.M. et al. (1991) *J. Biol. Chem.* **266**, 9023-9031). Due to their high-energy phosphate bonds they are utilized in rapid phosphotransfer reactions like the two-component

15   system and the phospho-relay signaling (West,A.H. & Stock,A.M. (2001) *Trends Biochem. Sci.* **26**, 369-376).

Most recently, histidine phosphorylation was identified to contribute to regulatory processes in vertebrates like stimulation of human platelets (Crovello,C.S. et al.

20   (1995) *Cell* **82**, 279-286) or regulating annexin I –function (Muimo,R. et al. (2000) *J. Biol. Chem.* **275**, 36632-36636). Another well established example is the regulative phosphorylation of ATP-citrate lyase by nucleoside diphosphate kinase (NDPK) (Wagner,P.D. & Vu,N.D. (1995) *J. Biol. Chem.* **270**, 21758-21764). However, protein phosphoamidases acting specific on phospho-amidates in

25   proteins like histidine phosphorylated proteins are rarely described. First evidence for suchlike phosphoamidase activity was found as additional function of PP1 and PP2A when acting on P-His histone 4 (Kim,Y. et al. (1993) *J. Biol. Chem.* **268**, 18513-18518; Kim,Y. et al. (1995) *Biochim. Biophys. Acta* **1268**, 221-228). Although histidine phosphate is known to be present in mammals (Crovello,C.S.

30   et al. (1995) *Cell*, **82**:279-286), it has not to date been possible to identify either the corresponding kinases or the relevant phosphoamidases. A protein with assigned specific phosphoamidase activity, recognizing amongst other substrates P-His-NDPK but not hydrolyzing O-phosphorylated substrates, was isolated from bovine liver but no sequence identity is published (Hiraishi,H. et al. (1999) *J.*

35   *Biochem. (Tokyo)* **126**, 368-374). Furthermore, a 17-kDa phosphoamidase that is specific only for phosphorylated Arg and is free of the activity that hydrolyzes O-

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5   phosphorylated compounds has been described (Kuba,M. et al. (1992) *Eur. J. Biochem.* **208**, 747-752; Yokoyama,K., et al. (1993) *J. Biochem.* **113**, 236-240 Kumon, A. et al. (1996) *J. Biochem.* **119**, 719-724).

A protein phosphoamidase specific for hydrolyzing N-phosphorylated histidine
10   residues in peptides or proteins and having no activity that hydrolyzes O-phosphorylated peptides or proteins is PHP1 (WO 00/52175; Ek,P. et al.(2002) *Eur. J. Biochem.* **269**, 5016-5023).

In scope to understand the physiological role of phosphoamidases and to be able
15   to interact with patho-physiological situations it is important to identify the enzyme-substrate specificity of a phosphoamidase unequivocally, and to develop tools to interfere by means of an inhibition or activation of the enzyme.

Within the meaning of the present invention the term "phosphoamidase" defines
20   an enzyme hydrolyzing phosphoamide (P-N) bonds of phosphorylated basic amino acids like P-His, P-Lys, P-Arg, or of peptides or proteins comprising these phosphorylated amino acids and which is devoid of an activity that hydrolyzes O-phosphorylated proteins or peptides. Examples for such phosphoamidases within the meaning of the present invention are described in Hiraishi,H. et al. (1999) *J.*
25   *Biochem. (Tokyo)* **126**, 368-374, Kuba,M. et al. (1992) *Eur. J. Biochem.* **208**, 747-752 and WO 00/52175.

Within the meaning of the present invention the term "protein phosphoamidase" defines a phosphoamidase hydrolyzing phosphoamide (P-N) bonds of
30   phosphorylated basic amino acids like P-His, P-Lys or P-Arg only within peptides or proteins and which is devoid of an activity that hydrolyzes O-phosphorylated proteins or peptides. An example for such a protein phosphoamidase within the meaning of the present invention is the protein histidine-phosphate specific phosphoamidase PHP1 described in WO 00/52175.

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5   Within the meaning of the present invention the term "protein histidine-
       phosphoamidase" defines a protein phosphoamidase specific for hydrolyzing
       phosphorylated histidine within peptides or proteins. An example within the
       meaning of the present invention is PHP1 as described in WO 00/52175.

10   Within the meaning of the present invention the term phosphatase defines an
       enzyme hydrolyzing phosphoester (P-O) bonds of phosphorylated amino acids
       like P-Thr, P-Ser or P-Tyr, or of peptides or proteins comprising these
       phosphorylated amino acids. These enzymes may have additionally a
       phosphoamidase activity.

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       Within the meaning of the present invention the term "protein phosphatase"
       defines a phosphatase hydrolyzing phosphoester (P-O) bonds of phosphorylated
       amino acids like P-Thr, P-Ser or P-Tyr only within peptides or proteins. These
       enzymes may have additionally a phosphoamidase activity. Examples for protein

20   phosphatases within the meaning of the present invention are PP1, PP2A and
       PP2C (Kim,Y. et al. (1993) *J. Biol. Chem.* **268**, 18513-18518).

       Within the meaning of the present invention the term "phosphoamidase activity"
       defines the hydrolyzation of a phosphoamide bond irrespective whether it is

25   hydrolyzed by a phosphoamidase or phosphatase.

       Within the meaning of the present invention the term "activity of a
       phosphoamidase" defines the hydrolyzation of a phosphoamide bond by a
       phosphoamidase, protein phosphoamidase or protein histidine phosphoamidase

30   which is devoid of an activity that hydrolyzes phosphoester (P-O) bonds of
       phosphorylated proteins or peptides.

       There is some literature on biochemical methods suitable for detection of
       phosphohistidine dephosphorylation. For example the malachite green method

35   (Ohmori,H. et al. (1993) *J. Biol. Chem.* **268**, 7625-7627; Ohmori,H. et al. (1994) *J.
       Biochem.* **116**, 380-385) has been used to identify phosphoamidase activity for

5 6-phospholysine and 3-phosphohistidine. Released P_i was assayed successive
   addition of malachite green and citric acid. The absorbance of the
   phosphate/molybdate complex was measured at 630 nm (Kuba,M. et al. (1992)
   Eur. J. Biochem. **208**, 747-752). However this assay may strongly interfere with
   the acid lability of physiological phosphoamidase substrates. 6-phospholysine, 3-
10 phosphohistidine and the corresponding dephosphorylated amino acids have
   been identified by thin layer electrophoresis on cellulose and staining with
   ninhydrin or Rosenberg's reagent.

   Using ^{32}P-labeled histidine-phosphorylated histone H4, the protein phosphatases
15 PP1, PP2A and PP2C have been shown to have a protein phosphoamidase
   activity for histidine in addition to their Ser-, Thr- or Tyr-phosphatase activity
   (Kim,Y. et al. (1993) *J. Biol. Chem.* **268**, 18513-18518; Matthews,H.R. &
   MacKintosh, C. (1995) *FEBS Lett.* 364, 51-54; Kim,Y. et al. (1995) *Biochim.*
   Biophys. Acta **1268** 221-228). Phosphoamidase activity for histidine was detected
20 by incubation of $[^{32}P]$histone 4 with phosphatase and subsequent acrylamide-
   electrophoresis of the reaction mixture and autoradigraphy of the gel. For
   quantitative analysis $^{32}P_i$ released from $[^{32}P]$histone 4 was removed by
   ultracentifugation, and the remaining histone-bound phosphate was quantified by
   liquid scintillation counting.
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   A further test on the activity of a protein His-phosphoamidase has been described
   in WO 00/52175 using ^{32}P-labeled histidine-phosphorylated CheA as substrate.
   CheA is a recombinant bacterial histidine autokinase (Bilwes,A.M. et al. (1999)
   Cell **96**, 131-134). Free phosphate has been identified by thin-layer
30 chromatography via ammonium molybdate or by autoradiography, respectively.

   A further possibility to detect inorganic phosphate has been described in WO
   95/02825 and Brune et al. 1994 (*Biochemistry* (1994) **33**, 8262-8271). This assay
   is based on the rapid shift in the fluorescence characteristics of MDCC-PBP,
35 which is the E. coli phosphate binding protein (PBP) labelled with N-[2-(1-

5   maleimidyl) ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC), a
     detectable fluorescent label.

     However, because the methods mentioned above can not be used in a
     continuous assay, or are time consuming and/or generate radioactive waste, they
10   are not well applicable in HTS (High Throughput Screening) runs. Therefore there
     is a need of further protein phosphoamidase and phosphoamidase assays which
     are easy to perform and applicable in HTS runs.

Summary of the invention

15   The present invention relates to the use of the compounds FDP (fluorescein
     diphosphate), DDAO (9H-(1,3-dichloro-9,9-dimethyl-acridin-2-one-7-
     yl)phosphate) DiFMUP (6,8-difluoro-4-methylum-belliferyl phosphate), ELF®39
     phosphate (2-(2′-phosphophenyl)-4-(3H)-quinazolinone) and ELF®97 phosphate
     (2-(5′-chloro-2′-phosphophenyl)-6-chloro-4-(3H)-quinazolinone) (all available
20   from Molecular probes, Inc., Eugene Oreg.) for the detection, characterization
     and qualitative and/or quantitative determination of the activity of phospho-
     amidases or protein phosphoamidases independent from the availability of a
     physiological substrate, which is often unknown for novel enzymes. Such
     compounds are of interest in relation to characterize the physiological role of
25   phosphoamidases or protein phosphoamidases and to identify their enzyme-
     substrate specificity unequivocally, and to develop tools to interfere by means of
     an inhibition or activation of the enzyme.

     In a further aspect, the invention relates to methods for the identification of a test
30   substance as an inhibitor or activator of phosphoamidases and/or protein
     phosphoamidases. Such a method may comprise the steps:
     a) establishing a sample comprising the phosphoamidase or protein
        phosphoamidase and a test substance,
     b) administering FDP, DDAO, DiFMUP, ELF®39 phosphate and ELF®97
35   phosphate to the sample,

5   c) detecting the signal produced by the substrate, and

     d) identifying the test substance as an activator or inhibitor of the

        phosphoamidase or protein phosphoamidase by comparing the signal

        produced in the sample comprising the test substance with the signal

        produced in a control sample comprising no test substance.

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Its another embodiment of the invention to provide methods for the detection of

phoasphoamidases on native gels, SDS-gels or blots thereof by incubating the

gels or blots with ELF®39 phosphate and/or ELF®97 phosphate after

electrophoresis and detecting the fluorescence produced by the substrates after

15   reaction with the enzymes for example by a standard tabletop UV-illuminator.

Furthermore the present invention provides methods to identifiy inhibitors or

activators specific for a distinct phosphoamidase in a sample comprising other

phosphoamidases or phosphatases by incubating the gels or blots after

20   electrophoresis with the substance to be tested and subsequently with ELF®39

phosphate and/or ELF®97 phosphate. The specifity of the inhibitor can be

determined by comparing the signals produced in the gel incubated with the test

substance with the signals in a control gel without test substance.

25   Other objects of the present invention are apparent for someone skilled in the art

on the basis of the following detailed description.

Brief description of the figures

30   **Figure 1:** pH-dependence of initial reaction rates of the conversion of fluorogenic

and chromogenic substrates by PHP1.

Figure 2: Time resolved measurement of phosphate released from phospho-

peptides by alkaline ohosphatase (AP) (left diagram) and PHP1 (right

35   diagram). The fluorescence signal is converted into phosphate

5   concentration using a normalizing factor derived from standard curve
       titration of inorganic phosphate with fluorescent phosphate binding
       protein.

Figure 3: Detection of phosphatase activity using the malachite green assay for
10   the reaction of LAR-PTP (left diagram) and of PHP1 (right diagram)
                with phospho-peptides.

Figure 4: Mass spectrometric analysis of the reaction product of incubating
                phosphopeptides with individual phosphatases/phosphoamidases. The
15   loss of 80 Da in mass indicates the enzymatic cleavage of phosphate
                from the pS-peptide by λPP and AP.

Figure 5: Mass spectrometric analysis of the reaction product of incubating
                phosphopeptides with individual phosphatases/phosphoamidases. The
20   loss of 80 Da in mass indicates the enzymatic cleavage of phosphate
                from the pT-peptide by λPP and AP.

Figure 6: Mass spectrometric analysis of the reaction product of incubating
                phosphopeptides with individual phosphatases/phosphoamidases. The
25   loss of 80 Da in mass indicates the enzymatic cleavage of phosphate
                from the pY1-peptide by Lar-PTP and AP.

Figure 7: Kinetic analysis of the conversion of DiFMUP by PHP1:
                a) Increase in fluorescence due to the dephosphorylation of DiFMUP
30   at various substrate concentrations (in μM). The increase in initial
                   fluorescence signal is due to the background fluorescence of
                   unreacted substrate.
                b) Correlation of relative fluorescence with the concentration of fully
                   converted substrate for quantification purpose.

c) Lineweaver-Burk plot of inversed initial rates versus inversed DiFMUP-concentration with linear fit.

d) Michaelis-Menten plot of initial reaction velocities versus substrate concentration. The linear correlation does not allow for non-linear curve fit deriving the Michaelis-Menten parameter.

Figure 8: Kinetic analysis of the conversion of FDP by PHP1:

a) Increase in fluorescence due to the dephosphorylation of FDP at various substrate concentrations (in µM). The increase in initial fluorescence signal is due to the background fluorescence of unreacted substrate.

b) Correlation of relative fluorescence with the concentration of fully converted substrate for quantification purpose.

c) Lineweaver-Burk plot of inverse initial rates versus inverse FDP-concentration with linear fit.

d) Michaelis-Menten Plot of initial reaction velocities versus substrate concentration. The hyperbola correlation does allow for non-linear curve fit deriving the Michaelis-Menten parameter.

Figure 9   Plot of inhibitor concentration vs. DifMUP-readout after 5 min reaction time at 0,1µM PHP1 concentration.

Figure 10:   Dot-blot with serial dilution of PHP1 (5 µg , 2.5 µg, 1.3 µg, 0.6 µg, 0.3 µg, 0.15 µg, 0.08 µg, 0.04 µg) and Lar-PTP (1 µg, 0.5 µg, 0.25 µg, 0.13 µg, 0.06 µg, 0.03 µg, 0.015 µg, 0.08 µg).

Figure 11:   a) Image of SDS-PAGEs after separation of I) AP, II) PHP1 and III) Lar-PTP (each 5 µg) and subsequent renaturation and soaking with fluorogenic substrates (ELF®39 phosphate and ELF®97 phosphate). The first lane was loaded with pre-stained protein marker, that exhibits auto-fluorescence.

b) Image of Western blots corresponding to the SDS-PAGEs shown in
Fig. 8a) after separation of a) AP, b) PHP1 and c) Lar-PTP (each
5µg) and subsequent renaturation and soaking with fluorogenic
substrates (ELF®39 phosphate and ELF®97 phosphate). The first
lane was loaded with prestained protein marker, that exhibits auto-
fluorescence.

Detailed description of the invention

In general, assays for the determination of protein phosphatase activity are
frequently based on the determination of released inorganic phosphate from
chromogenic or fluorogenic substrates.

Several phosphatases recognize mono- or diphosphate esters of small
fluorophore or chromophore compounds as artificial substrates, cleave the
phosphate ester bonds and thereby release the fluorophore or chromophore
respectively. These substrates have a fluorophor or chromophore with hydroxyl
moieties in common that form an ester bond with a phosphate group. They are
frequently used in phosphatase assays e.g. for alkaline phosphatase (AP) or
PP2A. The phosphatase activity is followed by the increased fluorescence or
absorption response of the reaction product. Due to different chemical properties
like the pKa of the phosphate ester, the substrates DiFMUP (6,8-difluoro-4-
methylum-belliferyl phosphate) (Gee,K.R. et al. (1999) *Anal. Biochem.* **273**, 41-
48), MUP (4-methylumbelliferyl phosphate) (Anthony,F.A. et al. (1986) *Anal.
Biochem.* **155**, 103-107; Rietz,B. & Guilbault,G.G. (1975) *Clin. Chem.* **21**, 1791-
1794), DDAO (9H-(1,3-dichloro-9,9-dimethyl-acridin-2-one-7-yl)phosphate)
(Leira,F. et al. (2000) *Toxicon* **38**, 1833-1844), FDP (fluorescein diphosphate)
(Huang,Z. *et al.* (1999) *J. Biomol. Screen.* **4**, 327-334), pNPP (para-nitrophenol
phosphate) and DCAP (Osawa,S. et al.(1995) *Clin. Chem.* **41**, 200-203) are
compatible with a broad range of phosphatases.

5   The protein histidine phosphoamidase PHP1 neither hydrolyzes phosphoester (P-
     O) bonds of phosphorylated proteins or peptides (see Figs. 2-6) nor it accepts the
     phosphate ester pNPP, a commonly used substrate of phosphatases like PP2A
     and acid phosphatase. Also MUP, a substrate of AP, PP1, PP2A and acid
     phosphatase is not accepted as substrate by PHP1 (see Fig.1). It was therefore
10   surprising that PHP1 accepts DiFMUP, a fluorinated derivative of MUP, FDP,
     DDAO, ELF®39 phosphate and ELF®97 phosphate (see Figs. 1 and 7 to 11) as
     substrates although these compounds are phosphate esters as well as pNPP and
     MUP.

FDP	DDAO phosphate
ELF39 phosphate	ELF97 phosphate
DiFMUP	MUP
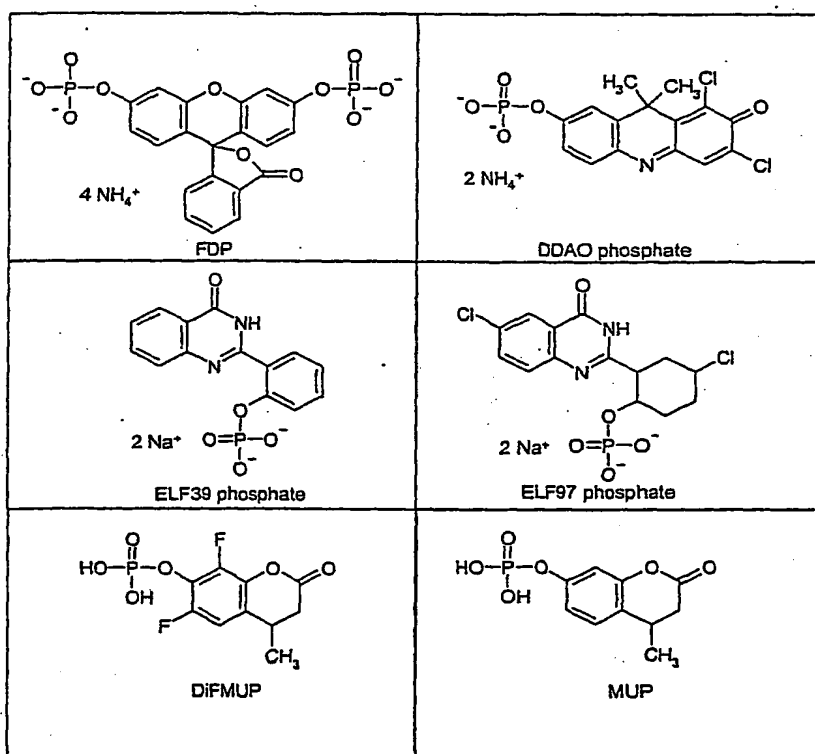
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     For example, figure 2 (left panel) shows the reactivity of AP with the commonly
     used and commercially available substrates for protein phosphatases, pY1
     (Sequence: RRLIEDAEpYAARG), pY2 (Sequence: TSTEPQpYQPGENL), pS
     (Sequence: RRApSVA) and pT (Sequence: KRpTIRR). For the detection of
20   phosphate released from the peptides the MDCC-PBP-assay has been used as

5   described in the examples. The phospho-tyrosine peptides pY1 and pY2 are
dephosphorylated by AP with a velocity (pmolPi/min) of 11 for pY1 and about 12
for pY2 and a specific activity (nmol/min * mg) of about 33 for pY1 and about 36
for pY2. To a smaller extend the phospho-serine peptide pS is dephosphorylated
with a velocity of 3 and a specific activity of 9. The phospho-threonine peptide pT
10   is not dephosphorylated by AP.

As shown in figure 3 (left panel), the protein tyrosine-phosphatase LAR (LAR-
PTP) dephosphorylates the phospho-tyrosine peptide but neither the phospho-
serine peptide nor the phospho-threonine peptide. In these experiments the
malachite green assay as described in the examples has been used for the
15   detection of phosphate released from the peptides. In strong contrast, using
PHP1 as enzyme (Figs 2 and 3, right panels) the dephosphorylating activity does
not exceed the blank value although the enzyme concentration was 30-fold
higher than that of AP and 40-fold higher than that of LAR-PTP.

20   These results were verified by the use of mass spectrometry (see Figs. 4-6),
which is a powerful tool to the identity of biopolymers by their individual masses.
The phospho-peptide substrates have masses of 700-1500 Da and will loose 80
Da upon cleavage of the phosphate group. This mass difference is easily
detected with high mass accuracy in a MALDI-TOF spectrometer. Moreover it is a
25   well accepted fact, that dephosphorylated peptides are more easily detected in
mass spectrometers than their phosphorylated counterparts (Sickmann,A. &
Meyer,HE (2001) Proteomics 1, 200-206). Hence even a small fraction of
converted peptides will be detectable if the substrate is accepted by the
respective phosphatase. Another advantage of this method is that the nature of
30   the substrate entity is directly monitored. Even MALDI-induced dephosphorylation
can be easily distinguished from enzymatic dephosphorylation in case of
phospho-serine and -threonine residues as the first will produce mass differences
of 98 Da instead of $\Delta m = 80$ Da in the latter case.

5   The mass spectrometric analysis show that the pS-peptide was recognized as
     phosphatase substrate by λPP and AP but neither by Lar-PTP nor by PHP1 (Fig.
     4). After incubation with either λPP or AP a major fraction becomes
     dephosphorylated within the reaction time and a species with a mass reduced by
     80 Da corresponding to the dephosphorylated peptide can be detected. The pS-

10   peptide incubated with Lar-PTP or either PHP1 does not cleave at all.
     The mass spectrometric analysis shows that the pT-peptide was recognised as
     phosphatase substrate solely by λPP but neither by Lar-PTP nor by PHP1 or AP
     (Fig. 5). After incubation with λPP a substantial fraction becomes
     dephosphorylated within the reaction time and a species with a mass reduced by

15   80 Da corresponding to the dephosphorylated peptide can be detected. The pT-
     peptide incubated with Lar-PTP, AP or by PHP1 does not change at all.

     In contrast to phospho-serine and –threonine residues the phospho-tyrosine
     peptide exhibits a substantial fraction of dephosphorylated species even in the

20   control sample. The origin of this species remains uncertain, as phospho-tyrosine
     may eliminate phosphoric acid under MALDI-conditions that would equal a loss of
     $\Delta m = 80$ Da. This is essentially the same mass difference observed after
     enzymatic cleavage.

25   The mass spectrometric analysis shows that the pY-peptide was recognised as
     phosphatase substrate by Lar-PTP and AP but neither by λPP nor by PHP1 (Fig.
     6). After incubation with either Lar-PTP or AP the peptide becomes completely
     dephosphorylated within the reaction time and a species with a mass reduced by
     80 Da corresponding to the dephosphorylated peptide can be detected. The pT-

30   peptide incubated with λPP or PHP1 does not change at all as compared to the
     control sample.

     In summary, in none of the reaction mixtures a phospho-peptide was accepted as
     a substrate for PHP1 but indeed for the other phosphatases tested with the

35   expected substrate specificities.

5   As a result of the finding that the compounds FDP, DDAO DiFMUP, ELF®39
       phosphate and ELF®97 phosphate are substrates for phosphoamidases and
       protein phosphoamidases like PHP1, the present invention provides easily
       executable continuous enzymatic assays for the determination of
10   phosphoamidases and protein phosphoamidases.

       Because it is well known that the use of phospho fluorophores like DiFMUP,
       MUP, DDAO or FDP as phosphatase substrates have certain limitations due to
       the compatibility of the substrate pKa-values with the pH-optima of the respective
15   phosphatase, the pH-dependence of the PHP1 reactivity towards the
       aforementioned fluorogenic substrates is to be determined. As shown in figure 1
       the pH is varied from pH = 5-9 in steps of 0.5 pH-units. In order to avoid
       undesired fluorescence flattening effects due to high absorption by the
       accumulating reaction product the initial reaction velocity is used as relative
20   measure for the pH-dependence of PHP1-reactivity towards the selected
       substrate. This approach gives reasonable results for MUP, DiFMUP and FDP
       but not for DDAO even at 25 µM dilution. The latter therefor may provide high
       sensitivity for trace detection of phosphatase activity, but may not be optimal for
       the use as a substrate above low µM-concentrations e.g. for studies of enzyme
25   kinetics.

       From this experiment a clear substrate selectivity of PHP1 towards the phosphate
       esters DiFMUP, DDAO, FDP as substrate becomes evident. As mentioned
       above, the most widely used artificial phosphatase substrate with broad
30   applicability pNPP as well as the typical alkaline phosphatase substrate MUP are
       not recognised and processed by PHP1 at any pH. The titration curves of the
       other substrates show very distinct shapes that may reflect both the pH-
       dependence of the enzymatic reaction and the chemical properties, like the
       protonation grade of the fluorophores. All three, DiFMUP, FDP and DDAO show
35   increasing turnover rates measured at their standard emission wavelengths when

5   the pH is shifted from acidic to neutral with a maximum at pH=6.5-7. The
     optimum pH for PHP1 is known to be at about a pH of 6 but an overlaying effect
     due to protonation of the fluorophore and shift in spectral properties is obvious
     from a colour change of all samples in acidic media. While DiFMUP reveals a
     decrease in turnover rate in more alkaline media DDAO turnover stays relatively
10   constant and the curve for FDP exhibits a second distinct maximum. The cause
     for the latter observations may originate from two populations of singly and
     doubly dephosphorylated FDP with individual spectral properties or substrate
     recognition by PHP1.

15   In further experiments the K_M-values for the reaction of PHP1 with DiFMUP and
     FDP have been determined (see Figs. 7 and 8). The K_M-values for DiFMUP and
     FDP can be approximated by recording the initial reaction velocity in dependence
     of the substrate concentration, as observed by the increase in relative
     fluorescence at the respective fluorescence emission maxima upon addition of
20   PHP1. The reaction velocity of the DiFMUP and FDP conversion by PHP1 is
     clearly dependent from the substrate concentration. From the titration
     experiments with fully converted substrate it becomes obvious, that signal
     saturation occurs within the low µM range, particularly for the fluoresceine
     derivative FDP. This effect limits the range of substrate variation. For FDP
25   concentrations >20 µM the signal quenching disturbs reaction rate measurements
     detrimentally. However data analysis using non-liner curve fit with a hyperbola
     function allows deducing a Michaelis constant of $K_M \approx 20$ µM with a high
     uncertainty. Measurements for DiFMUP are limited to concentrations below 500
     µM. Despite this higher range for substrate variation no saturation in initial
30   reaction rate could be observed as the correlation between rates and substrate
     concentration remained linear. From the Lineweaver-Burk plot the K_M-value might
     be estimated to exceed 1 mM. From literature K_M values of 20 µM are known e.g.
     for the enzymatic reaction of T-cell tyrosine phosphatase on DiFMUP (Kerby,M.
     et al. (2001) *Electrophoresis* **22**, 3916-3923). The higher value for the reaction of
35   PHP1 on DiFMUP might reflect the less accurate substrate acceptance of PHP1
     for phosphate ester substrates. In this regard FDP is not comparable to DiFMUP

5   as it bears two phosphate groups and this will certainly improve enzyme
    substrate interaction.

    In order to test whether the phosphoamidase and protein phosphoamidase
    substrates of the present invention are suitable for the for identification of
10 inhibitors or activators of these proteins, the activity of PHP1 was determined with
    the substrate DifMUP in presence of bisperoxo-(bipyridine)-oxovanadate
    [(bp)V(bipy)], a well characterized inhibitor of protein phosphotyrosine
    phosphatase(Fig. 9). The inhibitor concentration varied from 0.01 to 30 µM, the
    enzyme concentration was kept at 0.1 µM and substrate concentration at 50 µM.
15 After incubation for 15 min at room temperature the fluorescence was detected at
    450 nm on excitation at 360 nm. The assay yielded a reproducible and clearly
    dose-dependent inhibition of PHP1 activity for (bp)V(bipy). Surprisingly the
    (bp)V(bipy) compound activated the PHP1 activity at equimolar concentrations by
    a factor of two and showed an inhibitory effect only above 10 µM.

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    It becomes clear from these experiments that the phosphoamidase and protein
    phosphoamidase substrates FDP, DDAO DiFMUP, ELF®39 phosphate and
    ELF®97 phosphate are suitable for the identification of inhibitors or activators of
    these enzymes. These substrates allow the implementation of easily executable,
25 time saving and no radioactive waste generating enzymatic assays, also
    applicable in HTS runs.

    The compounds ELF®39 phosphate and ELF®97 phosphate are fluorogenic
    substrates for alkaline phosphatases designed for applications in immunoblots
30 and histological stains of enzyme activity. These substrates have the properties
    to be converted into an insoluble strongly fluorescent precipitate upon phosphate
    cleavage at the specific location of phosphatase activity. Usually AP is detected
    by the substrate conversion due to the high specific activity compared to other
    phosphatases and a wide substrate specificity of this enzyme.

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5   Unexpectedly PHP1 recognizes both fluorogenic substrates ELF®39 phosphate
     and ELF®97 phosphate as substrates. Figure 10 shows a dot blot of a dilution
     series of PHP1 and LAR-PTP after subsequent incubation with the ELF®97
     phosphate substrate solution.

10   As demonstrated in figure 10, PHP1 shows a distinct signal at enzyme quantities
     under 100 ng even though if a relatively insensitive detection set-up is used.
     The production of the fluorescent precipitate at the side of phosphoamidase
     activity in principle allows any kind of spatial resolution but does not give any clue
     to the nature of the phosphoamidase. The combination of the separation of
15   proteins by SDS-PAGE, native gel electrophoresis, 2D-electrophoresis or
     isoelectric focussing with the enzyme can circumvent this problem. Commonly
     enzyme activity is detected after electrophoresis by extracting the enzyme from
     the polyacryamide matrix or by converting substrates that have been co-
     polymerised in the polyacrylamide matrix or co-migrated into the gel with the
20   enzyme sample. Soaking the gel with small artificial substrates provides an
     alternative that is already used but with the frequent disadvantage that such
     substrate retain soluble and diffuse back from gel to bulk solution once they are
     converted. ELF®39 phosphate and ELF®97 phosphate represent unique
     substrates which change not only spectral properties but also their solubility and
25   therefore remain within the gel where the phosphatase or phosphoamidase is
     located. An important issue is that the phosphoamidases have to be renatured at
     least for the part of the catalytic domain after the denaturing SDS-PAGE or 2D-
     electrophoresis has been performed.

30   In the experiments presented in figure 11a) and 11b) samples of a) AP, b) PHP1
     and c) Lar-PTP (each 5 µg) have been separated on 10% NOVEX® NuPAGE®.
     Both, the SDS-PAGE and the western blots demonstrate that PHP1 was
     renatured after separation and was able to convert the fluorogenic substrates
     ELF®39 phosphate and ELF®97 phosphate into the desired fluorescent
35   precipitates. These precipitates accumulate at the location of the PHP1 protein

5   band in the SDS Page, with the expected apparent molecular weight of 14kDa
     (estimated from the protein marker and coomassie counter staining).

     With these substrates it is also possible to determine whether a certain inhibitor
     or activator acts specific on only one phosphoamidase or phosphatase in a
10   sample comprising other phosphoamidases or phosphatases or whether the
     inhibitor or activator inhibits or activates several phosphoamidases or
     phosphatases. For that, a sample comprising several phosphoamidases and/or
     phosphatases is separated for example by native electrophoresis. Subsequently
     the gel is equilibrated in a first step with the inhibitor or activator and in the
15   second step with ELF®39 phosphate and/or ELF®97 phosphate. The specifity of
     the inhibitor or activator can be determined by comparing the signal produced in
     the gel incubated with the inhibitor or activator with the signals in a control gel
     without test substance. For example, if the sample separated by gel
     electrophoresis contains five different phosphatases or phosphoamidases which
20   react with ELF®39 phosphate and/or ELF®97 phosphate, the gel will show five
     spots resulting from the dephosphorylation of the substrate. If a second gel is
     incubated with an inhibitor specific for only one of the phosphatases or
     phosphoamidases the subsequent incubation with ELF®39 phosphate and/or
     ELF®97 phosphate will result in only four spots. By comparing the gel containing
25   no inhibitor with the gel incubated with the inhibitor the specificity of the inhibitor
     can be evaluated. If instead of a native gel a SDS- or IEF-gel is used, it has to be
     assured that the proteins of interest have been renatured.

     ## Examples

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     ### Analysis of phosphatase/phosphoamidase activity
     In order to evaluate the substrate specificity of PHP1 towards other
     phosphorylated peptide sequences synthetic phospho-peptides have been
     selected as artificial substrates. These commercially available peptides (Upstate
35   Inc., Waltham, MA) are commonly used substrates for protein phosphatases that

5   dephosphorylate phospho-serine phospho-threonine or phospho-tyrosine with the
respective sensitivities:

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Name	Kind of phosphopeptide	Sequence	Source
pY1	Tyrosine phosphopeptide	RRLIEDAEpYAARG	Upstate Inc., Waltham, MA
pY2	Tyrosine phosphopeptide	TSTEPQpYQPGENL	Upstate Inc., Waltham, MA
PS	Serine phosphopeptide	RRApSVA	Upstate Inc., Waltham, MA
PT	Threonine phosphopeptide	KRpTIRR	Upstate Inc., Waltham, MA

The phosphatases used for comparative studies of substrate recognition are LAR-PTP (tyrosine phosphatase LAR, recombinant), λ-PP (protein phosphatase lambda, recombinant) and AP (alkaline phosphatase, bovine intestine).

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The enzyme concentrations are individually adjusted for every experimental set up in the fashion that enzymatic reactions can be monitored in a linear regime on the second to minute time scale.

15   a) MDCC-PBP-assay

In a first set of experiments the phosphatase reaction of PHP1 is compared with that of alkaline phosphatase using the above mentioned phosphopeptides as substrates. In order to follow the reaction continuously the phosphate entity that was cleaved of by the phosphatase is detected using the coumaryl labelled

20   phosphate binding protein MDCC-PBP (WO95/02825; Brune et al.(1994) *Biochemistry* 33, 8262-8271) which is available from the Medical Research Council, London, UK. In a first step the basal level of free phosphate in solutions of 40 µM phospho-peptide (in assay buffer: 25 mM Hepes pH 7.0, 1 mM MgCl₂, 20 mM NaCl, 0.01 mg/ml BSA) has to be determined by titration versus a solution

25   of 4 µM phosphate binding protein. Non of the peptide solutions exceeded 0.5 mol% of free phosphate/peptide. The phosphatase/phosphoamidase reaction was performed by mixing 50 µl of peptide solution (20 µM final concentration) with 50 µl 6 µM MDCC-PBP dilution in assay buffer in white microplates at room temperature and finally by adding 50 µl of the phosphatase or phosphoamidase

5   solution. The final concentration of the enzymes in the test solution are 0.035 μM
     for AP and 1 μM for PHP1. The increase in fluorescence (Ex 425 nm; Em 465
     nm) is measured time resolved using a standard microplate reader.

     b) Malachite green assay:

10   The enzymatic reaction of Lar-protein tyrosine phosphatase towards the
     phosphopeptides is compared to that of PHP1 using the classical malachite
     phosphate assay. Inorganic phosphate cleaved from the peptide substrate is
     detected as a green co-complex with molybdate and malachite dye. The
     phosphatase reaction is performed by mixing 950 μl of peptide solution (50 μM

15   final concentration) with 50 μl of the phosphatase/phosphoamidase solution, both
     stock solutions were prepared in standard assay-buffer (25 mM Hepes pH 7.0, 1
     mM MgCl$_2$, 20 mM NaCl, 0.01 mg/ml BSA;). The final concentration of the
     enzymes in the test solution are 0.025 μM for Lar-PTP and 1 μM for PHP1. After
     discrete time intervals a 50 μl sample of each reaction mixture was mixed with

20   the molybdate/malachite detection solution (BIOMOL) and incubated at room
     temperature. The release of phosphate was identified by the increase of
     absorption at 630 nm.

     pH-dependence of PHP1 assays

25   The reaction mixture was prepared by diluting the substrate from the 10 mM
     stock solution in DMSO into the aqueous reaction buffer consisting of 25 mM
     buffering substance, 20 mM NaCl, 1 mM MgCl$_2$, 0.1mg/ml BSA to yield a final
     concentration of 100 μM for MUP and DiFMUP or 25 μM for DDAO and FDP
     respectively. As buffering substance MES/NaOH was used for pH-values of 5.0,

30   5.5, 6.0, 6.5 whereas Tris/HCl was used for pH-values of 7.0, 7.5, 8.0, 8.5, 9.0.
     To start the reaction PHP1 was added from a pre dilution in the respective
     reaction buffer to yield a final concentration of 0.1 μM. The reaction was
     monitored by recording the increase in relative fluorescence at the respective
     fluorescence emission maxima (Fluoromax Gemini XL microplate reader).

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5   Kinetic analysis of the conversion of FDP and DiFMUP by PHP1

The reaction mixture is prepared by diluting the substrate from a 10 mM stock
solution in DMSO into the standard aqueous reaction buffer (25mM HEPES pH =
7.0, 20 mM NaCl, 1 mM MgCl$_2$, 0.01mg/ml BSA) to yield a final concentration
ranging from 1-400 µM. To start the reaction PHP1 was added from a stock

10 solution to a final concentration of 0.1 in µM. The reaction was monitored by
recording the increase in relative fluorescence at the respective fluorescence
emission maxima in an SLM fluorescence spectrometer. To allow for
quantification the fluorescence signal was normalised using a standard curve
recorded for a serial dilution of fully dephosphorylated DiFMUP and FDP

15 respectively. All recordings were carried out at the same PM-voltage and
integration time with correction for the relative signal gain.

Mass-spectrometric analysis of enzyme activity towards synthetic phosphorylated
peptides

20 The experimental setup is as follows: The phospho-peptide substrate is diluted
from stock-solutions to concentrations of 50 µM and mixed with the individual
phosphatase to give final enzyme concentrations of 1 µM PHP1, 0.05 µM Lar-
PTP, 0.05 µM λPP and 0.05 µM AP.

The mixtures is incubated for 2 h at room temperature and finally spotted in

25 volumes of 0.5 µl onto a MALDI-target. Briefly thereafter the matrix solution of
alpha-hydroxy-cinnaminic acid was added and the droplets were left to dry. Mass
spectrometry was performed on a MALDI-TOF spectrometer in reflective mode
with delayed extraction.

30 Screening assays for inhibitors or activators of protein phosphoamidases and
phosphoamidases.

The reaction mixtures for inhibitor screening were prepared by serial 2fold dilution
of the inhibitor bpV(bipy) (Alexis Biochemicals San Diego) starting at 60 µM into
50 µl of the assay buffer consisting of 25 mM Hepes pH=7, 20 mM NaCl, 1 mM

35 MgCl$_2$, 0.01mg/ml BSA. In a next step 50 µl of a solution of 0.3µM PHP1 in assay
buffer was added to each well and incubated for 5 min at room temperature. In

5   order to initiate the reaction a pre dilution of 50 μM DiFMUP-substrate in assay-buffer was rapidly added to all wells. The reaction was monitored by recording the increase in relative fluorescence at the respective fluorescence emission maxima (Fluoromax Gemini XL microplate reader). The relative activity was calculated using the RFU derived from a control reaction without inhibitor.

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<u>Detection of of protein phosphoamidases and phosphoamidases in blots and gels</u>
To show the utility of ELF®39 phosphate and ELF®97 phosphate for the detection of protein phosphoamidases and phosphoamidases PHP1 and LAR-PTP –are diluted in series by factor 2 with assay buffer (25mM HEPES pH 7.0, 20 mM

15   NaCl, 1 mM $MgCl_2$, 0.1mg/ml BSA) and 10 μl of each dilution is spotted on a nitrocellulose membrane. After a short wash (~5 min) in assay buffer the membrane was covered with a solution of the respective substrate (1:20 dilution of supplier stock-solution) in assay buffer and incubated for at least one hour or until a stable signal has developed. Fluorescence is detected on a standard

20   tabletop UV-illuminator (excitation wave length commonly 254 nm or 300-370 nm) or for higher signal selectivity by fluorescence imaging systems equipped with exciting light sources and narrow pass optical filters that allow optimal matching of the ELF®39 and ELF®97 spectral properties.
For the detection of protein phosphoamidase in SDS-Gels AP, PHP1 and Lar-

25   PTP (each 5μg) have been separated on 10% NuPAGE® BisTris/MES-gel system (Invitrogen®). Sample preparation was by adding reducing NuPAGE® SDS- sample buffer (Invitrogen®) to the enzyme solutions and immediate loading on the gel essentially without previous heating. After separation the gel was either used directly for detection or blotted onto nitrocellulose membrane using

30   NuPAGE® transfer-buffer (Invitrogen®) essentially without methanol. The gels and blots were than washed three times in Tris Buffered Saline, 1 mM $MgCl_2$ by gentle shaking for 30 min at room temperature and subsequently incubated with a solution of the respective substrate (1:20 dilution of supplier stock-solution) in assay buffer (25 mM HEPES pH 7.0, 20 mM NaCl, 1 mM $MgCl_2$, 0.1mg/ml BSA).

5   After incubation for at least one hour the fluorescence signal was detected as
        described above.

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